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Review article

Tetrapyrrole-based drought stress signalling

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Summary

Tetrapyrroles such as chlorophyll and heme play a vital role in primary plant metabolic processes such as photosynthesis and respiration. Over the past decades, extensive genetic and molecular analyses have provided valuable insights into the complex regulatory network of the tetrapyrrole biosynthesis. However, tetrapyrroles are also implicated in abiotic stress tolerance, although the mechanisms are largely unknown. With recent reports demonstrating that modified tetrapyrrole biosynthesis in plants confers wilting avoidance, a component physiological trait to drought tolerance, it is now timely that this pathway be reviewed in the context of drought stress signalling. In this review, the significance of tetrapyrrole biosynthesis under drought stress is addressed, with particular emphasis on the inter-relationships with major stress signalling cascades driven by reactive oxygen species (ROS) and organellar retrograde signalling. We propose that unlike the chlorophyll branch, the heme branch of the pathway plays a key role in mediating intracellular drought stress signalling and stimulating ROS detoxification under drought stress. Determining how the tetrapyrrole biosynthetic pathway is involved in stress signalling provides an opportunity to identify gene targets for engineering drought-tolerant crops.

Introduction

Global food security in the face of a changing climate demands increasing agricultural production on finite arable land without increasing water use. With predicted population increase to around 9 billion by 2050, the World Food Summit on Food Security (2009) sets a target of 70% increase in global food production. Rainfed agriculture will play a major role in meeting this demand as there is little opportunity for increasing irrigation schemes and many existing schemes are already under pressure. The single greatest abiotic stress factor that limits worldwide rainfed agriculture is drought. The need to breed crops better adapted to drought stress is an issue of increasing urgency. Drought tolerance is a quantitative trait, under highly complex genetic control (Fleury *et al.*, 2010; McWilliam, 1989). In the light of such complexities, the dissection and detailed understanding of individual pathways and processes that contribute to the various physiological mechanisms of drought tolerance is necessary.

Regulatory responses to drought stress

Plants have evolved complex signalling networks to sense and respond to drought stress. Such signalling cascades are composed of a suite of stress receptors, intercellular and intracellular signal transduction systems and transcriptional regulatory networks (Kuromori *et al.*, 2014). These drought-responsive signalling cascades can be triggered by diverse stimuli including osmotic shock, oxidative bursts, strong light, heat and wounding (Cruz de Carvalho, 2008; Wang *et al.*, 2003). Water deficit also leads to many cellular changes such as reduction in cell volume, disruption of inter- and intracellular water potential gradients, loss in cell turgor, disruption of membrane integrity, concentration of solutes and denaturation of proteins (Bray, 1997). Early recognition of these drought-induced cellular changes is the first step towards initiating plant acclimation responses. Abscisic acid (ABA) is a key stress-responsive phytohormone sensitive to these cellular changes, particularly to the loss of turgor (Schroeder *et al.*, 2001). Water deficit first triggers ABA biosynthesis in roots; ABA is then distributed throughout the plant via the transpiration stream (Shinozaki and Yamaguchi-Shinozaki, 2007). A series of recent genetic studies provide valuable insights into the molecular events from intercellular ABA perception to ABA-induced gene transcription. Increased cellular ABA concentrations are first detected by receptors such as pyrabactin resistance 1/PYR1-like/regulatory component of ABA response 1 (PYR/PYL/RCARs) (Ma *et al.*, 2009; Park *et al.*, 2009). Upon binding ABA, the receptor's conformation changes, leading to the activation of an ABA responsive element binding protein/ABRE-binding factor (AREB/ABF) (Shinozaki and Yamaguchi-Shinozaki, 2007; Umezawa *et al.*, 2010; Yamaguchi-Shinozaki and Shinozaki, 1994). This master ABA-responsive transcription factor regulates a diverse array of genes that coordinate cellular responses to the drought stress. Such cellular responses include stomatal closure, induction of stress proteins and accumulation of various metabolites for the protection of cells against water deficit stress (Kuromori *et al.*, 2014; Umezawa *et al.*, 2010). This ABA-dependent pathway is considered as a major component of the drought stress signalling cascade. Drought stress signals can also be propagated through ABA-independent pathways. Often these are a result of early osmotic stress-induced Ca^{2+} spiking/oscillation, which leads to calcium-dependent protein kinase (CDPK) activation and

drought-responsive gene transcription. Additionally, they can be a consequence of stress-responsive selective proteolysis or phospholipid hydrolysis (Schulz *et al.*, 2013; Zou *et al.*, 2010).

Another trigger for drought stress signalling is via the accumulation of ROS. Under steady state conditions, major plant metabolic processes including photosynthesis and respiration generate highly toxic ROS (Tripathy and Oelmüller, 2012). There are four types of ROS, namely singlet oxygen ($^1O_2$), superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO^-) (Cruz de Carvalho, 2008). To minimize potential cytotoxicity from ROS, plants have evolved efficient ROS detoxification mechanisms. When plants are exposed to stress, like drought and high light, the delicate equilibrium between ROS production and scavenging is perturbed (Cruz de Carvalho, 2008; Van Breusegem and Dat, 2006). ROS production is enhanced under drought stress due to limitations on CO_2 fixation and increased photorespiration. High concentrations of ROS are extremely deleterious and can cause severe photo-oxidative damage and cell death. However, low concentrations act as stress signals, triggering acclimation and defence mechanisms (Camp *et al.*, 2003). Rapid increases in ROS production (oxidative burst) and ROS generated through stress-induced metabolic imbalances have been shown to serve as stress signals (Mittler *et al.*, 2004). It has been reported that ROS activates Ca^{2+} channels and induces protein kinases and the expression of a suite of nuclear genes (Pei *et al.*, 2000; Pitzschke and Hirt, 2006; Pitzschke *et al.*, 2009). ROS is also implicated in interorganelle communication (retrograde signalling), which in turn activates related signal transduction pathways (Laloi *et al.*, 2007; Lee *et al.*, 2007). For comprehensive reviews on molecular mechanisms underlying drought stress signalling networks, refer to Bai *et al.* (2014), Baxter *et al.* (2014), Shinozaki *et al.* (2003) and Kuromori *et al.* (2014).

Although our knowledge of each signalling pathway is increasing, it is still difficult to develop a comprehensive picture of the multiple mechanisms governing drought stress signalling. Therefore, further investigations are required to discover how stress signalling pathways interconnect to form the major stress signalling cascades. The tetrapyrrole biosynthetic pathway has recently been implicated in wilting avoidance, a drought component trait (Allen *et al.*, 2010; Thu-Ha *et al.*, 2011). Based on detailed genetic and biochemical investigations, it has been proposed that tetrapyrrole biosynthesis is transcriptionally responsive to ROS-mediated stress signalling (Nagai *et al.*, 2007). An increasing body of evidence also suggests tetrapyrroles are involved in retrograde signalling. These signalling cascades work in concert to trigger stress-responsive gene expression. In this review, we outline the current knowledge linking tetrapyrrole biosynthesis to stress signalling as this may shed new light on molecular mechanisms important for enhancing drought tolerance.

Regulation of tetrapyrrole biosynthesis in plants

Tetrapyrrole biosynthesis is common to all higher plants and is responsible for the synthesis of chlorophyll, heme, siroheme and phytochromobilin which play vital roles in several primary metabolic processes (Tanaka and Tanaka, 2007). Mg^{2+}-containing chlorophyll, a cyclic tetrapyrrole, is the most abundant of plant tetrapyrroles. To date, five distinct chlorophylls, namely a, b, c, d and f, have been identified in photosynthetic organisms. As the major light-harvesting compound, chlorophyll plays a key role in photosynthesis, which converts light energy into chemical energy (Chen *et al.*, 2010). Similar to chlorophyll, heme is a cyclic compound, which contains Fe^{2+} instead of Mg^{2+}. Although chlorophyll is confined to plastids, heme has widespread cellular distribution. It is an important co-factor for many enzymes involved in respiration and ROS detoxification within chloroplasts, mitochondria and peroxisomes (Kirkman and Gaetani, 1984; Layer *et al.*, 2010; del Río, 2011). Siroheme, another Fe^{2+}-containing tetrapyrrole, is a prosthetic group to nitrite and sulphite reductases, which are involved in nitrogen and sulphur assimilation, respectively. Phytochromobilin is a linear tetrapyrrole synthesized in plastids and serves as the functional precursor of the phytochrome chromophore, which is involved in a wide range of processes including perception of red and far-red light (Kohchi *et al.*, 2001; Terry, 1997).

The tetrapyrrole biosynthetic pathway has been well described (Cornah *et al.*, 2003; Mochizuki *et al.*, 2010; Tanaka and Tanaka, 2007; Tanaka *et al.*, 2011), and two strict control points have been identified, each responding to tetrapyrrole demand. These two major regulatory points are (i) aminolevulinic acid (ALA) synthesis, and (ii) at the branch point between chlorophyll and heme synthesis (Figure 1).

Aminolevulinic acid is the universal precursor necessary for the synthesis of all tetrapyrroles. Therefore, ALA synthesis is tightly regulated both transcriptionally and post-translationally. The main enzyme-regulating ALA synthesis is glutamyl-tRNA-reductase (GluTR) (Tanaka *et al.*, 2011). In *Arabidopsis*, GluTR is encoded by three *hemin-deficient A* (*HEMA*) genes, which are differentially expressed across different tissues. They also each respond to distinct stimuli. For instance, *HEMA1* responds to a wide range of stimuli including cytokinins (Masuda *et al.*, 1995), light (McCormac and Terry, 2002; McCormac *et al.*, 2001) and circadian clock (Kruse *et al.*, 1997), plastid derived signals (McCormac *et al.*, 2001) and is highly expressed in photosynthetic tissues. In contrast, *HEMA2* expression is found exclusively in nonphotosynthetic tissues and is not responsive to illumination (Kumar *et al.*, 1996). The strong up-regulation of *HEMA2* under oxidative stress induced by ozone application and ROS-generating substances such as paraquat and rose bengal implies that *HEMA2* could play an important role in stress signalling and defence mechanisms (Nagai *et al.*, 2007). The third member, *HEMA3*, is lowly expressed, and its role is as yet not understood (Tanaka *et al.*, 1997, 2011).

Aminolevulinic acid synthesis is also regulated post-translationally by two important molecules, fluorescent (FLU) protein and heme. FLU is a nuclear-encoded plastid protein, which negatively regulates GluTR independently of heme, by binding to the C-terminal end of the enzyme. FLU specifically binds GluTR encoded by *HEMA1* (Meskauskiene and Apel, 2002). This negative regulation of ALA synthesis via FLU helps to prevent excessive accumulation of the highly photo-oxidative chlorophyll branch intermediate protochlorophyllide (Pchlide). Interestingly, Meskauskiene *et al.* (2001) demonstrated that inactivation of FLU-based negative regulation in the *flu* mutant enhanced Pchlide content but did not affect heme content. Therefore, the proposed effect of FLU is more likely to be restricted to the chlorophyll branch of the pathway (Meskauskiene *et al.*, 2001). Heme also exerts an inhibitory effect on GluTR activity by binding to its N-terminus. This was demonstrated by Vothknecht *et al.* (1998). Their study showed that a truncated GluTR, missing 30 amino acids at N-terminus, was highly resistant to feedback inhibition by heme *in vitro*. This was further supported in an *Arabidopsis long*

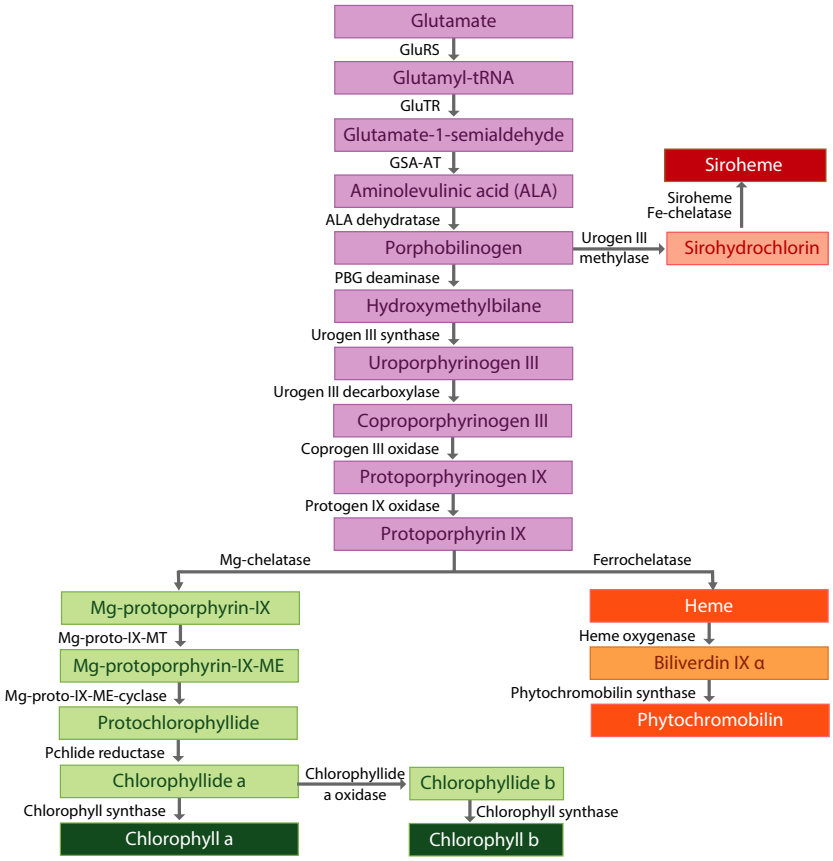

Figure 1 Tetrapyrrole biosynthetic pathway of higher plants, showing the major end products (white text in dark-coloured boxes) and catalytic enzymes. The common enzymatic steps, chlorophyll, heme and siroheme branches of the tetrapyrrole biosynthesis pathway are represented in purple, green, orange and red, respectively. GluRS, glutamyl-tRNA synthetase; GluTR, glutamyl-tRNA reductase; GSA-AT, glutamate 1-semialdehyde aminotransferase; Mg-proto-IX-MT; Mg-Protoporphyrin IX monomethylester.

hypocotyl (*hy1*) mutant, which showed deficiencies in heme oxygenase (HO) activity. HO is responsible for heme breakdown with *hy1* plants exhibiting a reduced rate of ALA synthesis and Pchlide content (Goslings *et al.*, 2004). Moreover, it has been proposed that several soluble proteins may associate with heme to exert its inhibition on GluTR (Srivastava *et al.*, 2005). However, the mode of action for both FLU and heme-regulated feedback is still not fully understood. How these negative regulatory mechanisms affect tetrapyrrole synthesis, particularly with regard to chlorophyll versus heme branch homeostasis under different physiological conditions, warrants future investigation. (Vothknecht *et al.*, 1998).

At the branch point, protoporphyrin IX (Proto IX) serves as the common substrate for both chlorophyll and heme branches. Insertion of Mg^{2+} into Proto IX by the enzyme Mg-chelatase (MgCh) favours the chlorophyll branch of the pathway, whereas insertion of Fe^{2+} by ferrochelatase (FC) leads to heme biosynthesis. The MgCh enzyme consists of three subunits namely, CHLH, CHLI and CHLD, with average molecular weights of 140, 40 and 70 kDa, respectively (Jensen *et al.*, 1996). The other requirements for the activation of this enzyme are an additional co-factor (Mg^{2+}), adenosine triphosphate (ATP) and a protein known as genomes uncoupled 4 (GUN4) (Davison *et al.*, 2005; Larkin *et al.*, 2003; Verdecia *et al.*, 2005). In contrast, FC is a single-subunit enzyme, which does not require a cofactor or an external energy source for catalysis (Al-Karadaghi *et al.*, 1997; Tanaka *et al.*, 2011). Studies on higher plants provide evidence for two FC isoforms (FC1 and FC2) that each fulfil distinct cellular functions. For instance, *FC1* is abundantly expressed in roots relative to leaves and stems (Chow *et al.*, 1998 Scharfenberg *et al.* 2014; Singh *et al.*, 2002; Suzuki *et al.*, 2002). Transcriptional gene fusions to β-*glucuronidase* have demonstrated that *Arabidopsis FC1* (*AtFC1)* promoter is induced in response to wounding, oxidative stress and viral infection (Singh *et al.*, 2002). Enhanced FC catalytic activity was also detected in chloroplasts of wounded leaves. This is further supported by subsequent studies, which demonstrated a marked induction of *AtFC1* expression in response to wounding, reagents generating ROS and drought stress (Nagai *et al.*, 2007; Scharfenberg *et al.*, 2014). In contrast, *AtFC2* was found to be expressed only within aerial parts of the plant, and its expression is markedly down-regulated or unchanged under the same treatments.

Previous studies indicate that during daylight, saturation with the tetrapyrrole precursor, ALA, causes a bias towards chlorophyll biosynthesis, whereas under darkness there is a bias towards heme biosynthesis (Cornah *et al.*, 2002). In the analysis of photodynamic changes in tobacco (*Nicotiana tobacum* L.), Papenbrock *et al.* (1999) demonstrated that ALA synthesis and MgCh activities increased during early light exposure, whereas FC activity was found to increase after a light-to-dark transition. This implies that cellular chlorophyll demand is higher during the day with a heme shift upon darkness. However, the extent of heme preference over chlorophyll biosynthesis and *vice versa* depends upon the plant developmental stage and its response to environmental stimuli. The dynamics of these changes in response to various physiological conditions, such as dehydration, is yet to be determined.

Tetrapyrrole biosynthesis activates ROS detoxification under stress conditions

Plants are constantly subjected to a wide range of environmental changes, which perturb cellular integrity and metabolism. Several studies have shown that tight regulation of tetrapyrrole biosynthesis becomes uncoupled upon exposure to stress conditions, leading to an over-accumulation of tetrapyrrole intermediates. Most tetrapyrrole intermediates including uroporphyrinogen III (Urogen III), coproporphyrinogen III (Coprogen III), Proto IX, Mg-protoporphyrin IX (Mg-Proto IX), Mg-protoporphyrin IX monomethylester (Mg-Proto IX ME) and Pchlide (Figure 1) act as strong photosensitizers (Cornah *et al.*, 2003) and generate the extremely strong oxidizing agent $^1O_2$, upon illumination. Even though this free radical is highly hazardous, tetrapyrrole intermediate accumulation seems to concomitantly trigger cellular protection and defence mechanisms. For instance, Urogen III decarboxylase (UROD) and Coprogen III oxidase (CPO) antisense tobacco plants exhibiting excess levels of Urogen III and Coprogen III showed enhanced resistance to viral infection (Mock *et al.*, 1999). These plants also displayed increased activity of stress-responsive ROS detoxification enzymes including superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX) (Mock *et al.*, 1998). It is interesting to note that, not only plastidal SOD, but both cytoplasmic and mitochondrial SOD activities are enhanced in these plants. As UROD and CPO are localized in plastids (Kruse *et al.*, 1995; Mock *et al.*, 1995; Smith *et al.*, 1993), this indicates that increased tetrapyrrole intermediates in plastids are able to trigger antioxidative responses throughout the cell. Whether these tetrapyrrole compounds actually leak into the cytoplasm and other subcellular compartments or whether they generate a rapidly transmissible intercellular signal to trigger this antioxidative response is unknown. As Urogen III and Coprogen III have not yet been detected in the cytoplasm or any organelle except in chloroplasts, we can rule out the former possibility. However, available evidence has led us to speculate that oxidative stress generated by tetrapyrrole intermediate accumulation is more likely to generate a rapid plastid signal that modulates nuclear gene expression implicated in antioxidative responses.

Enhanced tetrapyrrole biosynthesis is likely to confer drought tolerance via ROS detoxification

In recent years, the key tetrapyrrole precursor, ALA, has been extensively used to improve plant growth and stress tolerance in many plant species. It has been reported that exogenous ALA application enhanced chlorophyll content (Al-Khateeb *et al.*, 2006), photosynthetic rate (Wang *et al.*, 2004), antioxidant capacity (Balestrasse *et al.*, 2010), plant growth and yield (Al-Thabet, 2006). Such observations have been consistently noted under various stress conditions (salinity, drought and high temperature), in a variety of plant species including barley, wheat, rice, potato, soya bean, date palm, oilseed rape and cucumber (Li *et al.*, 2011; Liu *et al.*, 2011; Nishihara *et al.*, 2003; Zhang *et al.*, 2008). However, to date, only few studies have investigated the underlying molecular mechanisms for ALA promotion of dehydration tolerance. These few reports indicate that the application of 0.5–1 mg/L of ALA improved grain yield in wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare*) under drought conditions (Al-Thabet, 2006). ALA application at 0.1 and 1 mg/L concentrations also seems to promote chlorophyll biosynthesis, photosynthetic performance, biomass partitioning and ROS detoxification under water stress conditions (Li *et al.*, 2011). Strikingly, these plants exhibited low ROS production (H_2O_2 and O_2^-) when dehydrated, a likely consequence of increased activities of ROS-scavenging enzyme such as ascorbate peroxidase, catalase, GPX and SOD (Li *et al.*, 2011). Significantly increased chlorophyll content upon exogenous ALA application suggests that ALA either increases tetrapyrrole biosynthesis or inhibits chlorophyll degradation. In the scenario where tetrapyrrole biosynthesis is increased, there are most likely increased amounts of Proto IX that can be utilized by FC to generate heme-derived antioxidant biomolecules for defence. This could explain the observed increased activity of antioxidative enzymes upon exogenous ALA application. The study by Thu-Ha *et al.* (2011) supports this conclusion as they demonstrated the significance of the branch point intermediate, Proto IX, in dehydration tolerance. Transgenic rice plants overproducing Proto IX as a result of *Myxococcus xanthus PPO* overexpression appeared more tolerant to drought stress. These plants exhibited higher shoot water potential and leaf relative water content, less ROS production and higher ROS-scavenging enzyme activity when compared to wild-type plants. Transgenics were able to maintain higher ALA-synthesizing ability, through higher expression of *HEMA1* and glutamate-1-semialdehyde aminotransferase (*GSA*) upon dehydration, and they also showed significantly higher heme content, FC activity and expression of *FC2, HO1* and *HO2* both in leaves and in roots (Thu-Ha *et al.*, 2011). These observations show that increased ALA-synthesizing capacity and Proto IX levels lead to a bias towards the heme branch of the tetrapyrrole biosynthetic pathway. This proposed function of the heme branch in dehydration tolerance is further supported by experiments of Allen *et al.* (2010). By screening an *Arabidopsis* activation tagging (ACTTAG) population (100 000 lines) under water deficit conditions, they demonstrated that both *AtFC1* and *AtFC2* overexpression confer wilting avoidance. The overexpression of these *Arabidopsis* genes in maize also allowed plants to sustain yield upon water deficit, therefore further implicating the heme branch in drought stress signalling (Allen *et al.*, 2010). A more recent study by Kim *et al.* (2014) also provides weight to the role of heme in stress perception. Using transgenic rice plants ectopically overexpressing *Bradyrhizobium japonicum FC*, this study demonstrated increased cytosolic FC activity, increased total heme content, resistance to polyethylene glycol-induced osmotic stress as well as oxidative stress generated by peroxidizing herbicides.

Heme acts as an essential co-factor for ROS-scavenging enzymes such as SOD and catalase (Kirkman and Gaetani, 1984; del Río, 2011; Zhang and Hach, 1999). Not only heme, but also several other heme branch intermediates play important roles in ROS detoxification. It is well established that HO1 is a stress-responsive protein, which protects plants against oxidative damage induced by UV-B radiation (Yannarelli *et al.*, 2006) and H_2O_2 (Chen *et al.*, 2009; Jin *et al.*, 2013; Yannarelli *et al.*, 2006). Several recent studies provide evidence that HO1 is involved in stomatal closure induction (Cao *et al.*, 2007) as well as both lateral and adventitious root growth (Xu *et al.*, 2011; Xuan *et al.*, 2008). *HO1* is transcriptionally up-regulated in response to drought stress (Thu-Ha *et al.*, 2011; Wang *et al.*, 2014), implying that HO1 may play an important role in drought stress signalling. Furthermore, biliverdin IXα and carbon monoxide, products of heme breakdown by HO, also act as strong antioxidants (Barañano *et al.*, 2002; Han *et al.*, 2008; He and He, 2014; Stocker *et al.*, 1987).

Unlike the plastid-restricted tetrapyrroles, heme is capable of binding covalently and noncovalently to a large number of haemoproteins distributed across several cellular compartments (Espinas *et al.*, 2012). In addition to the involvement to ROS detoxification, in plastids, heme is an integral component of the cytochrome *b6f* complex, which is vital for electron transfer between photosystems I and II (PSI and PSII). To account for diverse functions outside plastids, heme must be either synthesized in different organelles or transported to individual cellular compartments. Heme as well as heme biosynthetic enzymes, such as protoporphyrinogen IX oxidoreductase (PPO) and FC, has been detected in purified fractions of chloroplasts and mitochondria of etiolated barley shoots (Jacobs and Jacobs, 1987, 1995). Interestingly, *in vitro* import assays have also shown that both FC1 and FC2 are localized to the stroma, thylakoid and envelope membranes of the chloroplast (Little and Jones, 1976; Masuda *et al.*, 2003; Papenbrock *et al.*, 2001; Roper and Smith, 1997) with FC1 additionally being imported into mitochondria (Chow *et al.*, 1997, 1998; Suzuki *et al.*, 2002). This may not reflect endogenous subcellular localization as subsequent *in vitro* import studies using purified pea and cucumber mitochondria exhibited undetectable FC1 activity (Lister *et al.*, 2001; Masuda *et al.*, 2003), whilst *in planta,* analysis of FC1 reporter proteins showed strict localization to the chloroplast (Lister *et al.*, 2001). To date, there is no *in planta* evidence showing FC1 localization to the mitochondria. These findings indicate heme biosynthesis is predominant in the plastids. We can also infer that heme is transported throughout the cell, given that haemoproteins can be found in many subcellular compartments.

Potential role of tetrapyrrole biosynthesis in intracellular drought stress signalling

Plant survival under harsh environmental conditions is primarily determined by the ability to avoid, escape or tolerate stress conditions. At the very early stage of drought stress, drought avoidance or acclimation strategies allow plants to minimize transpiration water loss via stomatal closure, adjusting leaf architecture, reducing leaf growth and shedding older leaves (Chaves *et al.*, 2009). Plants can also avoid dehydration by maximizing water uptake through accelerated root growth (Mundree *et al.*, 2002). Such adaptive alterations at the initial stages of water deficit stress can provide long-term protection from severe stress conditions. Some plants that exhibit developmental plasticity are able to escape drought by completing their life cycle before drought stress becomes lethal. Plants that contain increased levels of osmoprotectants such as proline, glycine, betaine and polyols are able to maintain turgor and protect cells from plasmolysis (Chaves *et al.*, 2009). Similarly, plants with high levels of antioxidants in response to dehydration can mitigate against ROS damage (Cruz de Carvalho, 2008). As outlined for ABA-dependent and ROS signalling, the induction of such drought adaptive strategies typically requires the perception of the dehydration stress, followed by inter- and intracellular stress signal transduction. Intracellular stress signalling cascades utilize secondary messengers for interorganelle communication, leading to stress-responsive gene transcription (Shinozaki and Yamaguchi-Shinozaki, 2007).

Among different cellular organelles, chloroplasts are known to be remarkably dynamic and highly sensitive to environmental cues. Photosynthesis is predominantly regulated in the chloroplast and is considered a global stress sensor (Biswal *et al.*, 2011). Light energy is the driving force for photosynthesis, and changes in its intensity are rapidly perceived by the photosensitive PSII complex (Biswal and Pessarakli, 2005; Biswal *et al.*, 2003). Water deficiency dramatically affects CO_2 fixation as a result of stomatal closure, which limits CO_2 uptake. This also leads to over reduction in the electron transport system within PSII and therefore problems with the dissipation of the absorbed light energy. This scenario ultimately causes significant redox imbalance and ROS generation, which consequently impairs the photosensor, PSII (Van Breusegem and Dat, 2006). A series of genetic and biochemical studies have revealed that these plastidal changes continuously signal to the nucleus to modulate gene expression via a process known as retrograde signalling. The existence of chloroplast-to-nucleus communication was first identified through a series of studies on chloroplast defective mutants as well as treatments with herbicides that affect chloroplast function such as norflurazon (NF). These studies revealed a marked reduction in nuclear gene expression of chloroplast-targeted proteins necessary for the assembly and functioning of the photosynthetic apparatus. This led Hess *et al.* (1998) to propose that functional chloroplasts are necessary for the expression of certain nuclear genes. This coordination process enables plastids to communicate chloroplastic demands, as a large number of plastidal proteins necessary for chloroplast biogenesis are encoded within the nuclear genome. These include nuclear-encoded polymerase, pentatricopeptide repeat proteins for RNA processing, photosynthesis-associated enzymes, and importantly all tetrapyrrole biosynthetic enzymes (Hedtke *et al.*, 2000; Pogson *et al.*, 2008; Tanaka and Tanaka, 2007).

Recent breakthroughs in understanding retrograde signalling have revealed novel pathways mediated under drought stress by 3′-phosphoadenosine 5′-phosphate (PAP) (Estavillo *et al.*, 2011) and methylerythritol cyclodiphosphate (MEcPP) (Xiao *et al.*, 2012). The identification of these compounds in plastidal signalling under stress conditions led the authors to speculate that plastids emit so-called operational signals to the nucleus specifically upon stress, to prevent and repair ROS damage. To date, a series of studies have revealed a large number of chloroplast-derived signalling molecules. These signals are generated by changes to chloroplast redox status and ROS accumulation (Kleine *et al.*, 2009). In the short term, cellular redox homeostasis is modulated by the plastoquinone (PQ) pool. Redox signals originating from imbalances of PQ abundance have been shown to regulate light-harvesting chlorophyll a/b binding protein (Lhcb) expression as well as light-harvesting complex II (LHCII) protein content (Foyer and Noctor, 2009; Yang *et al.*, 2001). Recent studies with *Arabidopsis* mutants reveal that ascorbate and glutathione also play a key role in this redox homeostasis and signalling to the nucleus (Ball *et al.*, 2004; Conklin and Barth, 2004; Schlaeppi *et al.*, 2008). However, the actual mechanisms for transferring the redox changes to PQ, glutathione and ascorbate pools remain elusive. Presently, the best candidate for a PQ-derived redox signal is state transition 7 (STN7), a thylakoid-localized LHCII protein kinase (Pesaresi *et al.*, 2009).

Reactive oxygen species has been implicated in operational signalling through studies with the *Arabidopsis* conditional *flu* mutant, which accumulates Pchlide upon darkness, a potent $^1O_2$ generator and photosensitizer (Camp *et al.*, 2003; Laloi *et al.*, 2007; Lee *et al.*, 2007; Wagner *et al.*, 2004). Affymetrix gene expression analysis by Camp *et al.* (2003) revealed etiolated *flu* seedlings, when exposed to light, rapidly activate the expression of 70 stress-responsive nuclear genes. It has also been reported

that excessive accumulation of $^1O_2$ in these seedlings suppresses photosynthesis-associated nuclear protein synthesis. Targets include small and large subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (RBCS and RBCL) and LHCB2 (Khandal *et al.*, 2009). Interestingly, thylakoid membrane-localized EXECUTER1 and EXCECUTER2 proteins appear to mediate the $^1O_2$-induced signalling cascade between the chloroplast and nucleus (Kim and Apel, 2013; Lee *et al.*, 2007). Singlet oxygen itself is unlikely to serve as a long distance signalling molecule given its highly reactive nature and short half-life. It has been suggested that $^1O_2$ may interact with neighbouring plastid components to generate more stable lipid-based metabolites, which could potentially serve as signalling molecules (Ramel *et al.*, 2012, 2013). H_2O_2 is proposed as a better signalling molecule because it is less toxic and has a longer half-life than $^1O_2$. Another candidate implicated in ROS-derived plastid signalling is β-cyclocitral, a product of $^1O_2$-induced oxidation of carotenoids. Importantly, β-cyclocitral has the capacity to induce a significant portion of the $^1O_2$-responsive genes, which in turn activate defence responses (Ramel *et al.*, 2012, 2013).

Heme-mediated chloroplast-to-nucleus signalling upon drought stress

It has been proposed that tetrapyrrole intermediates in both chlorophyll and heme branches are involved in chloroplast-to-nucleus communication during chloroplast biogenesis (Barajas-López *et al.*, 2013; Chi *et al.*, 2013; Kleine *et al.*, 2009; Surpin *et al.*, 2002; Terry and Smith, 2013). Even though previous studies in *Chlamydomonas reinhardtii* (Johanningmeier, 1988; Johanningmeier and Howell, 1984), garden cress (*Lepidium sativum*) (Oster *et al.*, 1996), *Arabidopsis* (Ankele *et al.*, 2007; Strand *et al.*, 2003) and barley (*Hordeum vulgare*) (La Rocca *et al.*, 2001) provided support for Mg-Proto IX being a retrograde signal, this concept was disputed in subsequent studies (Mochizuki *et al.*, 2008; Moulin *et al.*, 2008).

The evidence for the involvement of Mg-Proto IX in plastid signalling originated from studies on genomes uncoupled (*gun*) (Layer *et al.*) mutants. The *gun* mutants which are deficient in heme oxygenase (*gun2*), phytochromobilin synthase (*gun3*), MgCh-interacting protein (*gun4*) and CHLH (*gun5*) subunits displayed continuous expression of *Lhcb*, even when chloroplast development is impaired by the herbicide NF (Mochizuki *et al.*, 2001; Susek *et al.*, 1993). In all *gun* mutants, Mg-Proto IX content was drastically reduced, and this was interpreted as showing that this compound is an essential negative signal responsible for mediating nuclear gene expression. However, subsequent detailed analyses were unable to show a correlation between Mg-Proto IX content and degree of nuclear gene expression (*Lhcb*) in a range of *Arabidopsis* mutants grown under varying conditions (Mochizuki *et al.*, 2008; Moulin *et al.*, 2008). Furthermore, a Mg-Proto IX-accumulating barley *xantha-l* mutant did not demonstrate a reduction in nuclear gene expression (Gadjieva *et al.*, 2005).

Interestingly, in a detailed biochemical analysis, Voigt *et al.* (2010) demonstrated that in wild-type plants as well as *gun1*, *gun2*, *gun4* and *gun5* mutants, unbound free heme content was significantly increased upon NF treatment. Subsequent studies revealed that unlike Mg-Proto IX, heme is more likely to be the primary tetrapyrrole-based plastidal signal that modulates nuclear gene expression. For instance, Woodson *et al.* (2011) demonstrated that an *Arabidopsis gun* (*gun6-1D*) mutant overexpressing *FC1* induces *photosynthesis-associated nuclear* gene (PhANG) expression by increasing a specific heme subpool. Interestingly, overexpression of *FC2* is unable to enhance PhANG expression, implying that FC2-derived heme is less likely to be associated with retrograde signalling. This hypothesis was further confirmed by a recent study using *Arabidopsis sigma factor 2* (*sig2*) and *sigma factor 6* (*sig6*) (Woodson *et al.*, 2013). SIG is responsible for chloroplast transcription and the recognition of a number of tRNA promoters by plastid-encoded RNA polymerase (PEP) (Kanamaru and Tanaka, 2004; Kanamaru *et al.*, 2001). Mutants lacking *SIG2* and *SIG6* are deficient in PEP-transcribed $tRNA^{Glu}$, which is a precursor for tetrapyrrole biosynthesis, and a substrate for GluTR. Consequently, these plants show a reduction in $tRNA^{Glu}$, GluTR, ALA and Pchlide levels (Figure 1), as well as PhANG expression. However, overexpression of *FC1* in *sig2* and *sig6* mutant backgrounds was shown to restore PhANG expression, implying that heme is likely to be an important primary positive retrograde signal. Again, the overexpression of *FC2* in the *sig2* mutant background failed to increase PhANG expression (Woodson *et al.*, 2013). This was further supported by the observation that long hypocotyl mutants, *hy1* and *hy2* which accumulate heme and biliverdin IX due to impairment of HO and phytochromobilin synthase, displayed elevated nuclear gene expression upon exposure to NF (Vinti *et al.*, 2000). Even though the involvement of tetrapyrrole biosynthesis in operational signalling has yet to be fully established, there is existing evidence that leads us to speculate that this pathway may transiently generate a positive heme-based stress signal necessary for modulating nuclear gene expression under adverse conditions.

A proposed model for heme action as a retrograde signal leading to stress-activated gene expression

The proposed role for heme as an operational signal in chloroplast-to-nuclear signalling can be broken down based on the timing of molecular events. In the first instance, tetrapyrrole biosynthesis may favour heme production upon stress. Persistence of the stress event in this case would cause unbound free heme to accumulate and promote its efflux from the chloroplast. This would make more heme available for import to the nucleus. Once in the nucleus, heme may act to stabilize and activate specific transcription factor classes that bind to drought-responsive promoters. Transcriptional activation of drought-responsive genes would then lead to acclimation to the prevailing drought stress.

Under stress conditions, tetrapyrrole biosynthesis is perturbed, leading to the accumulation of intermediates (Mock and Grimm, 1997; Strand *et al.*, 2003). Given that this intermediate accumulation within the chloroplast significantly improves ROS detoxification enzymatic activity throughout the cell (Mock *et al.*, 1998, 1999), it is reasonable to assume that a stress signal might be transmitted from the chloroplast. Considering the literature available, we speculate that this stress signal is heme. For instance, it has been reported that, when tetrapyrrole flux is enhanced either by exogenous ALA application or by increasing Proto IX content, total heme content increases (Espinas *et al.*, 2012) upon drought stress (Li *et al.*, 2011; Thu-Ha *et al.*, 2011). Plants with increased total heme content show enhanced resistance to drought and oxidative stress (Kim *et al.*, 2014; Thu-Ha *et al.*, 2011). Tetrapyrrole intermediate accumulation within the chloroplast (Moulin *et al.*, 2008; Mundree *et al.*, 2002;

Van Breusegem and Dat, 2006) might additionally be a direct source of oxidative stress, which may in turn reinforce channelling of heme precursors towards heme production. Preferential channelling towards heme production is also supported by a study by Czarnecki *et al.* (2011) who showed that *Arabidopsis* GluTR-binding protein [GluTRBP; previously called proton gradient regulation 7 (PGR7)], when silenced, does not change ALA-synthesizing capacity or chlorophyll content but does reduce heme content. Further investigations are necessary to elucidate the mechanistic trigger for this process under stress.

It has been proposed that only unbound free heme, present in very small amounts relative to total heme, is important in retrograde signalling (Terry and Smith, 2013; Woodson *et al.*, 2011). Free heme quantification techniques are imprecise; therefore, little is known of the changes that occur in the free heme pool, particularly in response to stress. By combining different extraction techniques (Espinas *et al.*, 2012), it was determined that free heme content increases in wild-type seedlings upon NF-induced oxidative stress (Espinas *et al.*, 2012; Voigt *et al.*, 2010). This contrasts with total heme content, which actually decreases upon NF treatment (Espinas *et al.*, 2012; Woodson *et al.*, 2011), implying that when chloroplasts experience oxidative cytotoxicity, a portion of the covalently bound heme may also be released to the free heme pool (Espinas *et al.*, 2012). It is important to note that heme analysis in these various experiments was typically conducted a few days after the stress event, and therefore, rapid transient heme changes upon stress are currently unknown. To determine whether oxidative stress causes the accumulation of a transient free heme signal, during a complex event such as drought, precise time-resolved heme profiling will be needed. New approaches are necessary to elucidate the timing of tetrapyrrole changes following such stress events.

Heme is hydrophobic, and it is exported from the chloroplast to the cytoplasm (Severance and Hamza, 2009; Thomas and Weinstein, 1990). However, free heme molecules are considered cytotoxic as they are able to react with oxygen to produce ROS (Kumar and Bandyopadhyay, 2005). It has been proposed that due to low solubility of heme in aqueous solution, free heme is more likely to adhered nonspecifically to heme-trafficking proteins (Espinas *et al.*, 2012; Thomas and Weinstein). A large number of heme transporters have been identified in mammalian cells, as compared to plants where only a few have been identified (Krishnamurthy *et al.*, 2004; Quigley *et al.*, 2004; Severance and Hamza, 2009; Shayeghi *et al.*, 2005). A candidate for heme transport in plants is the translocator protein known as tryptophan-rich sensory protein (TSPO) (Balsemão-Pires *et al.*, 2011). In plants, TSPO is localized in the membranes of multiple organelles such as chloroplast, mitochondria, endoplasmic reticulum and the Golgi stacks (Balsemão-Pires *et al.*, 2011; Guillaumot *et al.*, 2009; Lindemann *et al.*, 2004). TSPO has a high affinity to heme (Vanhee *et al.*, 2011) and is translocated between subcellular compartments under abiotic stress conditions (Balsemão-Pires *et al.*, 2011). Therefore, TSPO is considered a likely candidate for heme transport across organellar membranes as well as a transporter throughout the cytoplasm under stress (Balsemão-Pires *et al.*, 2011; Taketani *et al.*, 1995). In addition, *Arabidopsis* heme-binding protein 5 (AtHBP5) has been identified as a chloroplast-localized protein which contains hydrophobic heme-binding pockets (Lee *et al.*, 2012). There are also a number of cytosolic localized heme carrier proteins, which transport heme between cellular organelles. In mammalian cells, a wide array of such proteins have been identified, including heme carrier protein 1 (HCP1), feline leucemia virus subgroup C cellular receptor (FLVCR) and ATP-binding cassette, subfamily G, member 2 (ABCG2) (Krishnamurthy *et al.*, 2004; Quigley *et al.*, 2004; Shayeghi *et al.*, 2005). Recently, several studies have shown that cytosolic AtHBP, homologous to mammalian heme-binding proteins p22HBP/SOUL, binds cytosolic heme (Sato *et al.*, 2004; Takahashi *et al.*, 2008; Zylka and Reppert, 1999). The presence of such a large number of heme carrier proteins supports the proposition that heme is more suitable as a signalling molecule than other tetrapyrroles.

It has been reported that in the nucleus, heme could post-translationally activate specific transcription factors that modulate a large number of genes necessary for stress acclimation. This proposition is based on studies conducted in yeast, where heme was shown to post-translationally activate the heme-responsive transcriptional regulator, HAP1. HAP1 is a nuclear localized protein, which exists in a high molecular weight complex in the absence of heme. This high molecular weight complex is composed of several heat-shock proteins including HSP90, HSP70, suppressor of RHO3 protein 9 (Sro9) and yeast dnaJ (Ydj1) (Hon *et al.*, 2001, 2005). In the presence of heme, HAP1 binds to heme via a conserved heme-responsive motif 7. This leads to the dissociation of Sro9 and Ydj1 from the complex resulting in the complete activation of HAP1. The resulting stable dimeric HAP1 complex has a high binding affinity to the DNA *cis*-element CGGnnnTAnCGG (Zhang and Guarente, 1994). The transcriptional activation of nuclear genes by the HSP70–HSP90–HAP1–heme complex is important for controlling oxidative damage in yeast. So far, a similar HAP1 complex has not been identified in plants. However, it was recently determined that HSP90 is essential for modulating nuclear gene expression in *gun5* upon oxidative stress (Kindgren *et al.*, 2012). *Arabidopsis* HSP90 is localized in the cytosol, chloroplast, mitochondria, endoplasmic reticulum and nucleus (Hubert *et al.*, 2009; Krishna and Gloor, 2001). If we suppose that the retrograde signal generated in *gun5* is heme, it would imply that heme–HSP90 interaction is necessary for activating nuclear gene expression. Interestingly, both *Arabidopsis* HSP70 and HSP90 molecular chaperones were found to be important for stomatal closure under drought stress conditions (Clément *et al.*, 2011). Taken together, it is probable that a similar mechanism in plants could initiate plant drought acclimation in response to oxidative stress.

In addition to HAP1, yeast contains another HAP2:3:4:5 transcriptional regulator complex which is post-translationally activated by heme. This complex triggers the transcription of a large number of genes through binding to CCAAT *cis*-elements (Maity and de Crombrugghe, 1998; Mantovani, 1998). *Arabidopsis* NF-YA:B:C complex members have been identified as orthologues of the yeast Hap2:3:4:5 complex (Stephenson *et al.*, 2007). NF-Ys are heme-activated heterotrimeric complexes composed of NF-YA, NF-YB and NF-YC subunits (Stephenson *et al.*, 2007). Importantly, the *cis*-elements targeted by this complex are found in the promoters of several drought-responsive genes (Li *et al.*, 2008). A series of studies have demonstrated that *Arabidopsis* NF-Y is involved in drought tolerance via both ABA-dependent and ABA-independent mechanisms (Nelson *et al.*, 2007; Stephenson *et al.*, 2007). For instance, *Arabidopsis* plants overexpressing *NF-YA5* were more resistant to drought stress due to prevention of water loss via ABA-induced stomatal closure (Li *et al.*, 2008). Furthermore, transgenic *Arabidopsis* and maize plants over-expressing *AtNF-YB1* and *ZmNF-YB2,* respectively,

exhibited drought tolerance phenotypes in an ABA-independent manner. These plants were less wilted and maintained higher leaf water potential, chlorophyll content, stomatal conductance, photosynthesis rate and yield under water-limited field conditions (Nelson *et al.*, 2007).

Several studies demonstrated that genes associated with tetrapyrrole biosynthesis (Stephenson *et al.*, 2010) are also activated by NF-Y transcription factors. Direct evidence linking tetrapyrrole biosynthesis with transcriptional regulation by NF-Y's comes from wheat transgenics overexpressing *NF-YB3,* which exhibited increases in the expression of *GluTR*, chlorophyll content and rate of photosynthesis under nondroughted conditions (Stephenson *et al.*, 2011). Moreover, an Affymetrix genome array showed that wheat *NF-YC11* and *NF-YB3* transcription factor genes co-express with light-inducible tetrapyrrole genes encoding GluTR, CHLH subunit and UROD (Stephenson *et al.*, 2010, 2011). Interestingly, *GluTR,* among other light-responsive genes, contains CCAAT-box motifs in their promoters (i.e. within 500 bp of translation start site), which is typical for NF-Y-binding *cis*-elements (Stephenson *et al.*, 2010). As GluTR is the first rate-limiting enzyme for tetrapyrrole biosynthesis, such evidence would suggest that tetrapyrrole biosynthesis might be transcriptionally regulated by NF-Y (Figure 2). In-depth analysis is necessary to elucidate how NF-Y-mediated transcriptional regulation could impact on tetrapyrrole biosynthesis under nonstressed as well as drought stress conditions.

Concluding remarks and future perspectives

Research efforts have indicated that tetrapyrroles are implicated in drought stress tolerance via retrograde signalling and induction of drought-responsive gene expression. It is evident that the tetrapyrrole pathway is favoured towards heme production upon water deficit stress and this triggers acclimation mechanisms (Figure 2). Even though the primary regulatory points of this pathway are known, the full set of molecular mechanisms facilitating dehydration tolerance still need to be identified. Some fundamental questions remain unanswered: What triggers the channelling of tetrapyrroles towards heme branch under stress? Is this in response to oxidative stress or do interacting proteins induce it? What influences heme efflux and its interorganelle transport upon stress? and Does heme activate nuclear gene expression via NF-Y transcription factors in plants?

It is important to note that recent studies have suggested the existence of two physiologically distinct heme pools, of which only one is involved in stress defence responses (Nagai *et al.*, 2007; Scharfenberg *et al.*, 2014; Singh *et al.*, 2002; Woodson *et al.*, 2011, 2013). It has been proposed that the heme pool involved in stress defence is likely generated through the action of HEMA2 and FC1, given that the genes encoding these enzymes are each transcriptionally activated upon oxidative stress as opposed to the *HEMA1* and *FC2* genes which are transcriptionally repressed (Nagai *et al.*, 2007; Singh *et al.*, 2002).

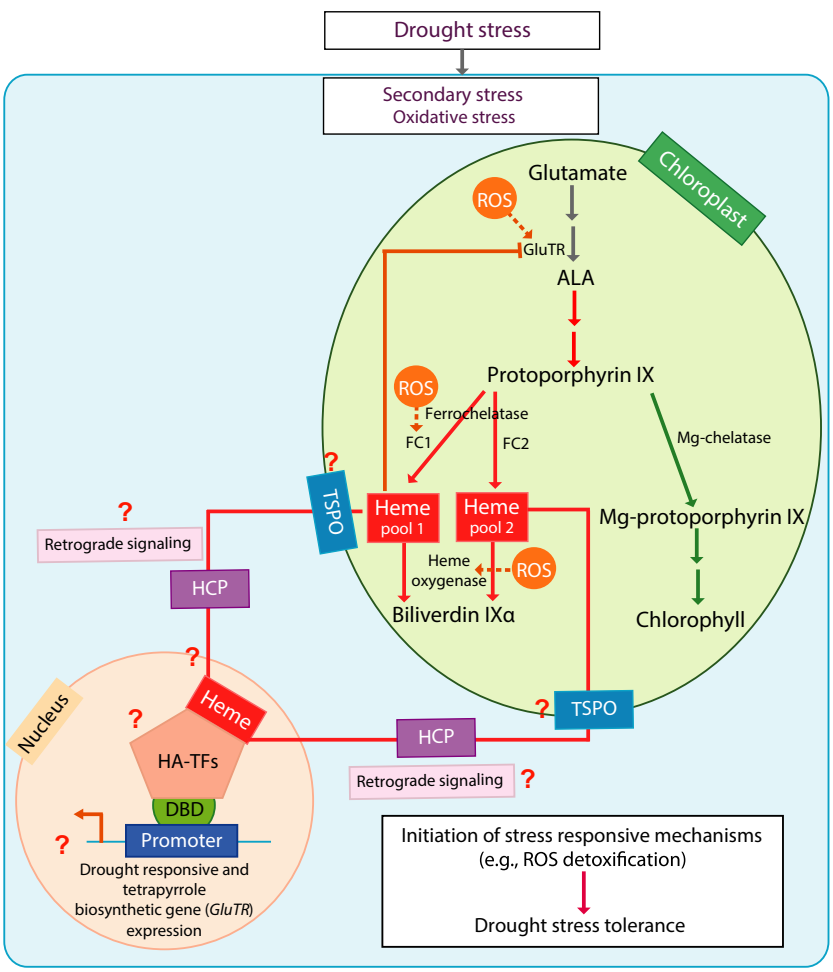

Figure 2 Proposed model based on current knowledge on the role of tetrapyrroles in drought stress signalling. Drought stress induces secondary stress events including chloroplast-localized oxidative stress, which in turn favours heme production. This enhances accumulation of unbound free heme, the plastid signal, for chloroplast-to-nuclear communication. Because free heme is insoluble and cytotoxic, its mobility is likely to be dependent upon both membrane and cytosolic localized heme carrier proteins (HCP) and transporters such as TSPO. Upon arrival in the nucleus, heme would post-translationally activate heme-activated transcription factors (HA-TFs) including the nuclear factor Y (NF-Y) class of transcription factors. We propose GluTR, encoding the first rate-limiting enzyme of the tetrapyrrole pathway, along with a suite of drought-responsive and reactive oxygen species (ROS) detoxification genes to be targets for this transcriptional activation. Heme-induced transcriptional activation would initiate and reinforce ROS detoxification, an important mechanism allowing plants to adapt to the prevailing drought stress. Dashed arrows indicate ROS transcriptionally induce genes encoding tetrapyrrole enzymes. Red question marks denote mechanistic points warranting further investigations.

Supporting this notion is the finding that *Athema2* and *Atfc1* loss-of-function mutants produce significantly less total heme upon oxidative stress when compared to wild type (Nagai *et al.*, 2007). Such a clear distinction between heme subpools should be taken with caution given that Scharfenberg *et al.* (2014) recently demonstrated that *fc2* but not *fc1* improves salt and oxidative stress tolerance. However, the proposal that distinct functions exist for the two heme subpools is supported by the finding that only FC1-derived heme seems to be involved in retrograde signalling (Woodson *et al.*, 2011, 2013). Future investigations are necessary to dissect the role of these potential heme subpools and whether they contrast in their effect on stress defence responses.

Another important area of research will be development of sensitive assays to precisely quantify free heme. Even though new techniques for measuring free heme have emerged (Espinas *et al.*, 2012), they remain somewhat imprecise. Thorough time-resolved quantifications are necessary to elucidate changes in total vs free heme upon drought stress. Moreover, appropriate protocols are yet to be developed for quantifying other intermediates of the heme branch, such as biliverdin IXα and phytochromobilin. The presence of these important intermediates in relatively low quantities make their analysis extremely difficult.

Despite these current limitations, our understanding on the contribution of tetrapyrrole biosynthesis in drought stress signalling will be useful for directing future research aimed at unravelling gene targets for engineering drought-tolerant crops.

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